Journal of Medical Virology 59:356–363 (1999)

HIV-1 in Semen: Determination of Proviral and Viral Titres Compared to Blood, and Quantification of Semen Leukocyte Populations

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This study was carried out to determine the relationship between proviral DNA and viral RNA titres in semen compared with blood. In addition, the association between semen leukocyte counts with detection frequency and absolute levels of human immunodeficiency virus type 1 (HIV-1) nucleic acids was also assessed. Paired samples of blood and semen were collected from a cohort of individuals with different blood CD4 cell counts, and whose anti-HIV therapy had not changed in the preceding 3 months. The cell-associated proviral DNA titres and cell-free plasma viral RNA titres were determined using nested primer polymerase chain reaction and NASBA™, respectively. In addition, leukocyte counts were determined by immunocytochemical and cytochemical staining of a subset of semen samples. HIV-1 proviral DNA was detected in 100% and 47%, and viral RNA was detected in 76% and 63%, of blood and semen samples tested, respectively. HIV-1 proviral DNA and viral RNA titres in blood were higher than in corresponding semen samples, although the difference observed in viral RNA titres was not statistically significant. Proviral DNA and viral RNA titres were correlated between the two body fluids, and within the semen, although some individuals had disparate semen and blood titres or detection rates, indicating genital tract compartmentalisation. In addition, detection of HIV-1 proviral DNA, but not of HIV RNA, in semen was associated with elevated semen leukocyte counts, although this latter finding requires verification in future studies of larger numbers of patients. ***J. Med. Virol. 59:356–363, 1999.***

KEY WORDS: HIV-1; genital tract; semen; viral RNA titres; proviral DNA titres

INTRODUCTION

Since the advent of blood and blood product screening, sexual transmission has been the main cause of new human immunodeficiency virus type 1 (HIV-1) infections worldwide. The underlying mechanisms involved in sexual transmission are relatively complex and a number of host and viral determinants might influence this process [Dean et al., 1996; Zhu et al., 1996; Bomsel, 1997; Anderson et al., 1998; Blaak et al., 1998; Dyer et al., 1998; Quayle et al., 1998]. Viral RNA is frequently detectable in semen during all stages of disease, and the absolute levels of cell-free virus can be as high as those observed in plasma [Gupta et al., 1997; Coombs et al., 1998; Dyer et al., 1998]. Concurrent genital tract infection is directly related to elevated viral RNA and proviral DNA titres [Mayer and Anderson, 1995; Atkins et al., 1996; Cohen et al., 1997; Xu et al., 1997; Fiscus et al., 1998]. This increase is presumably caused by leukocyte infiltration together with increased viral replication and viral receptor and coreceptor expression via elevated cytokine release [Anderson et al., 1998]. Undoubtedly, recent studies have focused attention on factors affecting viral RNA titres, and this attention might be due in part to the commercial availability of robust molecular detection methods for HIV-1 RNA. However, the possible role of cell-associated virus in sexual transmission should not be underestimated. Several lines of evidence suggest that cell-associated virus might have an important role in transmission. HIV-1 DNA is detected frequently in semen cells and plasma samples obtained from individuals at all stages of disease, and in vitro studies of the infectivity of different fractions of semen, together

Grant sponsor: The Medical Research Council, UK.

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Accepted 8 February 1999

with experimental inoculation of simians with both HIV-1 and the simian immunodeficiency virus (SIV), show that the infectious titres of cell-associated virus are higher than cell-free virus [Bourinbaiar and Phillips, 1991; Miller, 1992; Milman and Sharma, 1994; Joag et al., 1997; Coombs et al., 1998].

There is some evidence from in situ polymerase chain reaction (PCR) studies that spermatazoa contain proviral DNA [Nouvo et al., 1994]. However, results obtained from cell sorting experiments, which showed that proviral DNA was located exclusively in CD4-positive T cells and in cells of the monocyte/macrophage cells [Quayle et al., 1997], contradict this evidence. Importantly, the relative number of different haemapoietic cell subsets, and viral titres, varies widely between individuals and also over time in the same patient. These fluctuations are probably associated with disease stage, blood CD4 cell count, and concurrent genital tract infections [Wolff and Anderson, 1988; Cohen et al., 1997; Anderson et al., 1998; Fiscus et al., 1998].

Considering the potential importance of either the cell-associated and/or cell-free HIV reservoirs in the process of sexual transmission, there is a need to determine the relationship between viral RNA and proviral DNA titres in semen and blood. This knowledge would help in understanding of the underlying processes of sexual transmission of HIV, and might also influence the development of rational treatments designed to prevent this route of infection. To address this shortfall, we carried out a cross-sectional comparative study in which the proviral DNA and viral RNA titres in paired samples of blood and semen were determined. In addition, the leukocyte cell numbers in a subset of the semen samples were also measured, to assess how these numbers might be related to the HIV-1 titres.

MATERIALS AND METHODS

Source of Samples

Samples of peripheral blood and semen were collected from a cohort of 34 individuals with various blood CD4 cell counts who had not had any alteration of their antiretroviral therapy within the preceding 3 months. Semen and 5-ml blood samples collected over ethylenediamine tetraacetic acid (EDTA) were processed within 6 hr of collection. The total semen and blood volumes were determined using calibrated test tubes (Sarstedt, UK). A 250-μl aliquot of semen was diluted with an equal volume of RPMI 1640 medium containing 2% formaldehyde and then used for cell counting and immunocytochemical staining as described below. Blood and seminal plasma were prepared by centrifugation at 1,500 × *g* for 20 min, and resulting cell pellets were resuspended in 10 ml of RPMI 1640 medium. Washed semen cells (SFCs) were isolated by further centrifugation at 1,500 × *g* for 20 min. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient centrifugation of the resuspended blood cell pellet. The recovered PBMCs were then washed once with 15 ml RPMI 1640 medium, and the cells pelleted by centrifugation at 1,500 × *g* for 10 min. SFC and PBMC pellets were resuspended in 100 μl of RPMI 1640 medium, divided into two equal aliquots, then stored at −70°C for further use.

Immunocytochemical Staining

Total semen cell counts in the fixed semen samples were carried out using a Neubauer counting chamber. Percentage and total cell populations were determined using morphological characterisation and immunocytochemical staining of acetone-fixed cytospin preparations using monoclonal antibodies raised against human CD45, CD2, CD4, CD8, CD22, and the activated monocyte/macrophage antibody BerMAC3. Bound antibody was detected by mouse-specific streptavidin-conjugated secondary antibody, followed by alkaline phosphatase-conjugated biotin and chromogen. In addition, the number of granulocytes was determined by the presence of chloracetate esterase activity as detected by staining with naphthol AS-D. The specificity of immunocytochemical staining was assessed by omission of primary antibody. In addition, antigen integrity in fixed semen was demonstrated by mixing normal peripheral blood leukocytes with normal human semen, fixing and then detected as above. No loss of activity was observed.

Quantification of HIV-1 RNA Titres

Viral RNA titres were determined using the NASBA™ (Organon Teknika; UK) technique as described by the manufacturer using a 50-μl sample of semen or blood plasma as input. The number of copies per sample was calculated by comparison to the assay's standard curve, as described previously [van Gemen et al., 1993]. The cut-off for the assay using a 50-μl input was equivalent to 2,000 virus particles per millilitre. A 50-μl input of plasma was selected because larger volumes of semen were often associated with reactions in which the Qc calibrator value was discarded. This was most often caused by amplification failure of the Qc standard.

DNA Extraction

DNA was extracted from one stored aliquot of PBMCs and SFCs (equivalent to one-half of the total recovered cells) using the lysis and protein precipitating solutions from a commercially available genomic DNA extraction kit (Stratagene, UK). Cell lysis was carried out in the presence of 10 μg/ml of proteinase K (Sigma, UK) for 18 hr at 37°C. After extraction, the DNA was resuspended in 50 μl of sterile distilled water and the yield and overall quality of the extracted DNA was assessed by spectrophotometric analysis at 260 and 280 nm, as described previously [Sambrook et al., 1988]. The efficiency of the DNA extraction method was assessed by comparison of the actual and predicted DNA yields, which assumed that 150,000 diploid nucleated cells would yield 1 μg of DNA [Simmonds et al.,

1990], for a subset of samples. The estimated average extraction efficiency was 45% (range 30–58%) (not shown).

A cell lysis solution without dithiothreitol was used to minimise the amount of DNA recovered from spermatozoa [Giusti et al., 1986]. However, to assess whether spermatozoa DNA could contribute to the overall DNA yield, control extractions were carried out using Percol gradient-purified spermatozoa. Spermatozoa counts were performed using a Neubauer counting chamber and purity was assessed by microscopic examination. Five samples containing between 4 and 55 million spermatozoa were processed and, as expected, none of the extracts contained detectable amounts of DNA (not shown).

Quantification of HIV-1 Proviral DNA Titres

Two-microliter aliquots of two-fold dilutions of the extracted DNA samples were then used as templates in PCRs designed to amplify a fragment of HIV-1 encompassing V1 through V3 to determine the end-point dilution. Amplification was achieved using a nested primer PCR method yielding products of approximately 930 and 870 bp in the first and second rounds, respectively. The primers used were: G1, outer, sense, 5′GCC TGT GTA CCC ACA GAC CCC AA3′; 401_{dU}, inner, sense, 5′GAG GAU AUA AUC AGU UUT AUU3′; 308, outer, antisense, 5′ATT ACA GTA GAA AAA TTC CCC3′; and 307, inner, antisense, 5′CTG GGT CCC CTC CTG AGG3′. The first round PCRs were carried out in buffer consisting of 10 mM Tris-HCl pH 9.8, 50 mM KCl, 2 mM $MgCl_2$, 0.25 units of *Taq* polymerase, and 5 pmol of each primer G1 and 308 in a 25-μl reaction volume using 35 cycles of 94°C for 45 sec, 50°C for 45 sec, and 70°C for 3 min; 1 μl of this reaction was then used as template in equivalent asymmetric second round reactions using primers 401_{dU} with 307 for 30 cycles, followed by UNG treatment and a further 15 cycles to produce single-stranded DNA [Ball and Curran, 1997]. Unincorporated nucleotides were removed by treatment with 1 unit SAP (Amersham International, UK) for 15–30 min at room temperature followed by inactivation of the SAP by heating to 70°C for 15 min. The HIV proviral DNA titre was determined by amplification of at least 10 aliquots of DNA at the end-point dilution. In samples in which HIV-1 DNA could not be detected in the dilution series, 40 replicate nested primer PCRs using 1 μl of extracted DNA were carried out. The proviral titre per microlitre of extracted DNA was calculated according to the Poisson distribution formula $t = -\ln(F \times D)$, where t is the titre expressed as copies per microlitre of extracted DNA, F is the frequency of negative PCR reactions in the replicate series, and D is the extracted DNA dilution factor [Simmonds et al., 1990]. The proviral titre per millilitre of original sample (T) was estimated by correcting this value for the extract and original sample volume used and the average extraction efficiency using the formula $T = 50t(1/0.45)(1000/v)$, where t was the HIV DNA copy number per microlitre, v was the original blood/semen sample volume used for the DNA extraction, and the multiplication factors of 50 and 1/0.45 were used to correct for the DNA resuspension volume and extraction efficiency, respectively. The lowest semen sample volume was 1.1 ml (2 × 0.425-ml aliquots plus 0.25 ml removed for immunocychemistry), therefore, the assay detection limit was equivalent to less than 6 copies of HIV-1 DNA per millilitre when carrying out 40 replicate PCRs using 1 μl of undiluted DNA per reaction.

To verify that single molecules of HIV-1 proviral DNA were amplified routinely using the nested primer PCR approach, and therefore to ensure the sensitivity and quantitative nature of the assay, second round amplification products obtained at the end-point were sequenced directly. Aliquots of DNA containing single HIV-1 variants could be isolated from a complex mixture, such that individual sequence mutations between the distinct variants could be determined (not shown). This is consistent with a sensitivity for the amplification process of one copy per reaction for both semen and blood samples.

Verification of the Presence of Amplifiable DNA in HIV-1 DNA Negative Samples

Samples in which HIV-1 proviral DNA could not be detected were subjected to a single primer β-globin PCR, using primers described previously: GH21 (5′GGA AAA TAG ACC AAT AGG CAG3′) with PC03 (5′ACA CAA CTG TGT TCA CTA GC3′) [Saiki et al., 1988]. Cycling parameters were 30 cycles with temperature and time profiles equivalent to those used for the *env*-specific PCR. Products were resolved using agarose gel electrophoresis with ethidium bromide staining, as described previously [Sambrook et al., 1988].

Statistical Analyses

All tests used were nonparametric methods, and results below the assay detection limits for viral RNA and proviral DNA quantification were assigned values of 1999 and 5 copies per ml, respectively. Correlations were determined using the Spearman's Rank Correlation and differences between paired sample titres were assessed by Wilcoxon's Signed Rank. Differences in detection frequencies and in population medians were assessed using Fisher's exact test and Mann–Whitney's *U*-test, respectively. All tests were carried out using the Prism for Windows software package (Chilwell Scientific, Oxford, UK).

RESULTS

Semen Cell Counts

Total nucleated cell counts, together with immunocytochemical staining of specific leukocyte subsets, were determined for formalin-fixed semen samples obtained from some of the patient cohort for whom semen and blood HIV-1 proviral and viral titres were determined. Leukocytes, granulocytes, and macrophages were detected in all 19 samples tested, whereas CD4,

TABLE I. Cellular Composition of Semen

	Detection frequency	Median ($\times 10^5$ cells ml^{-1})	Range ($\times 10^5$ cells ml^{-1})
Round cells	19/19	14.60	1.60–124.00
Leukocytes (CD45)	19/19	4.34	0.05–34.72
CD4 lymphocytes (CD4)	10/19	0.14	ND–7.09
CD8 lymphocytes (CD8)	8/19	ND	ND–2.48
Macrophages (BerMac3)	19/19	1.17	0.02–4.96
B Lymphocytes (CD22)	7/19	ND	ND–1.74
Granulocytes (CAE)	19/19	1.10	0.04–13.64
SFMCs[a]	19/19	2.08	0.01–21.27
HIV host cells[b]	19/19	1.55	0.02–10.42

CAE, chloracetate esterase activity; HIV, human immunodeficiency virus; ND, not detected.
Antigen-specificities of the primary antibodies used in the immunocytochemical staining are shown in parentheses.
[a]Semen mononuclear cells calculated by subtracting the granulocyte cell count from the total leukocyte cell count for each sample.
[b]Calculated by adding the total number of CD4 lymphocytes and macrophages for each sample.

CD8, and B lymphocytes were detected in only 10, 8, and 7 samples, respectively (Table I). Of the possible HIV-1 host cells, macrophages were present in general at higher absolute numbers than CD4 lymphocytes (Wilcoxon's paired sign test $P < .005$) with median values of 117,000 (range 2,000–496,000) and 14,000 (range not detected–709,000), respectively.

Proviral DNA and Viral RNA Quantification in Blood and Semen Samples

A total of 32 paired blood and semen samples were obtained from the cohort of HIV-1 antibody-positive men. The proviral DNA and viral RNA titres were determined by limiting dilution PCR and NASBA™, respectively, and the results obtained are summarised in Table II. Proviral DNA was detected in 31/31 (100%) and 15/32 (47%), with median values of 496 (range 9–5,678) and less than 6 (range <6–2,171) copies per millilitre in blood and semen samples, respectively. PCR amplification of a fragment of the human β-globin gene was successful for all DNA extracts in which HIV-1 DNA was not detected, indicating that the inability to detect HIV-1 provirus in these samples was not due to the presence of PCR inhibitors (not shown). Viral RNA was detected in 26/34 (76%) and 19/30 (63%), with median values of 18,600 (range <2,000–977,600) and 5,600 (range <2,000–667,800) copies per millilitre in peripheral blood and semen plasma samples, respectively. Detection of HIV-1 proviral DNA in semen samples was associated with a concomitant detection of viral RNA in semen ($P < .05$; Fisher's exact test). Proviral DNA and viral RNA titres were higher in blood samples compared with the corresponding semen samples, with Wilcoxon's rank sum P values of < .0001 and .0653 for paired DNA and RNA titres, respectively. To assess whether the observed higher proviral titres in blood compared with SFCs were due to disparate numbers of individual or total host HIV-1 cells, proviral titres were corrected for the number of mononuclear cells and CD4 T cells present in each body fluid. Even after this adjustment blood proviral titres were significantly higher than those present in corresponding semen samples, irrespective of whether expressed as copies per 10^6 mononuclear white blood cells ($P < .01$), or per 10^5 CD4 positive T cells ($P < .005$). Although in general the blood proviral DNA titres were higher than the corresponding semen samples, in 2/19 patients this trend was reversed. In addition, in 5 patients RNA was detected at levels of at least 0.5 $\log_{10}$ times the assay cut-off, in only the blood plasma samples, whilst one patient had detectable HIV-1 RNA in the semen sample but not the corresponding blood sample.

Relationship Between Semen and Blood HIV-1 Nucleic Acid Titres and Blood CD4 Cell Count

Next, we determined what factors were associated with the detection of HIV nucleic acids (Table III). Blood and semen proviral titres were significantly higher in patients with a blood CD4 cell count below 200/μl compared with those with a blood CD4 cell count of greater than 200/μl, and blood proviral titres were also elevated in patients whose semen sample was positive for HIV-1 DNA. Although the viral RNA titres present in semen and blood were also elevated in these two groups of patients, the differences were not statistically significant. By contrast, blood viral RNA titres were significantly higher in patients whose semen samples were positive for HIV-1 RNA.

To investigate further the possible associations between DNA titres, viral RNA titres, and peripheral blood CD4 cell counts, Spearman's rank correlation coefficient tests were carried out. Semen and blood proviral titres were both weakly inversely correlated to peripheral blood CD4 cell counts, with Rs values of −0.3683 ($P < .05$) and −0.3508 ($P < .1$), respectively. In addition, the proviral titres present in the two sample sites were also correlated ($Rs = 0.3860$, $P < .05$). By contrast, there was no correlation between blood CD4 cell counts and either semen or blood plasma viral RNA titres. A strong positive correlation existed between the blood and semen viral RNA titres ($Rs = 0.5156$, $P < .005$). Finally, the semen viral and proviral titres were positively correlated ($Rs = 0.3841$, $P < .05$), but those for blood were not.

Relationship Between Semen Cell Counts and Disease Stage and HIV-1 Nucleic Acid Titres

To assess the possible role of host factors on the detection of HIV nucleic acids, we compared the leukocyte

TABLE II. Detection Rate and Titre of HIV-1 Nucleic Acids in Samples of Blood and Semen

	Frequency of detection	Median copies	Range
Blood proviral DNA (per ml)	31/31 (100%)	496	9–5,678
(per 10^6 white blood cells)		493	7–4,387
(per 10^5 CD4 cells)		198	2–8,867
Semen proviral DNA (per ml)	15/32 (47%)	<6	<6–2,171
(per 10^6 white blood cells)[a]		ND	ND–5,656
(per 10^5 CD4 cells)[b]		ND	ND–1,096
Blood viral RNA ($\times 10^4$ per ml)	26/34 (76%)	1.86	<0.20–97.76
Semen viral RNA ($\times 10^4$ per ml)	19/30 (63%)	0.56	<0.20–66.78

HIV, human immunodeficiency virus; ND, not detected.
Titres are expressed as total copies. HIV-1 DNA per ml blood > semen, $P < .0001$; HIV-1 DNA per 10^6 white blood cells blood > semen, $P < .01$; HIV-1 DNA per 10^5 CD4 cells blood > semen, $P < .005$; HIV-1 RNA per ml blood > semen $P = .0653$; (Wilcoxon's paired sign tests).
[a] $n = 19$, as semen cell counts had not been determined for all samples.
[b] $n = 16$, as CD4 cells were only detected in 10 of the 19 individuals for whom semen cell counts had been determined.

cell counts in HIV nucleic acid-positive and -negative samples, and also between individuals with either greater than or less than 200 CD4 cells per microlitre of blood. There was no significant difference (Mann–Whitney *U*-test) in the numbers of each leukocyte cell subset in semen samples obtained from individuals with greater than 200 compared to those with less than 200 blood CD4 cells per microlitre of blood (Table IV). However, the numbers of each of the leukocyte cells determined were higher, although not significantly, in semen samples that were HIV DNA positive compared with those that were HIV DNA negative. This result contrasted with the finding that leukocyte numbers were generally lower in HIV RNA-positive compared with HIV RNA-negative semen samples, although again these differences were not significant.

DISCUSSION

Most new infections of HIV-1 worldwide are acquired sexually, and consequently there has been a gradual increase of information relating to the size and composition of HIV-1 populations present in the genital tract. However, previous studies determined the relationship of either viral RNA titres or proviral DNA titres in blood compared with semen, but none so far have quantitated both the cell-associated and cell-free virus populations in the same patients. Therefore, this study was carried out to address this shortfall by determining the relationship, if any, between semen and blood viral and proviral loads. In addition, the cellular composition of semen within a subset of these patients was also analysed to assess its possible role in determining the size of the viral population.

The detection rate for HIV-1 proviral DNA and viral RNA in samples of peripheral blood was 100% and 76%, respectively, whilst for semen the detection rates were 47% and 63%, respectively. The blood proviral DNA detection rate is comparable to values reported previously [Simmonds et al., 1990; Xu et al., 1997], whilst the detection rate of proviral DNA in the semen samples was lower than that reported elsewhere [Mermin et al., 1991; Xu et al., 1997]. The presence of PCR inhibitors in the extracted samples was not a likely cause for this discrepancy as all the samples contained amplifiable human β-globin DNA. Also, unlike seminal plasma [Dyer et al., 1996], there are no previous reports of the presence of PCR inhibitors in the cellular fraction of semen [Mermin et al., 1991; Xu et al., 1997]. However, the decreased detection rate could have reflected the low efficiency (45% average) of the DNA extraction method we used. Unfortunately, we are not able to confirm or reject this hypothesis, as efficiencies for the extraction methods used in the other studies were not estimated. Detection rates of viral RNA in both blood and semen were also slightly less than those described elsewhere [Liuzzi et al., 1996; Gupta et al., 1997; Coombs et al., 1998], and this difference probably reflects the lower sample input used in our study. As stated previously, this approach was adopted to avoid decreased accuracy with low titre samples, as larger volumes of seminal plasma were often associated with amplification failure of the NASBA Qc standard.

The absolute HIV-1 nucleic acid titres present in semen and blood were in general comparable to those reported in other studies using similar quantitative methods [Mermin et al., 1991; Liuzzi et al., 1996; Gupta et al., 1997; Xu et al., 1997; Coombs et al., 1998]. Both the proviral DNA and viral RNA titres per millilitre were significantly higher in blood than in semen, and in contrast to a previous report [Xu et al., 1997], the proviral DNA titre was also higher in blood, even when equilibrated for the number of white blood cells or CD4 lymphocytes present in semen.

It was also apparent that the disease stage of the patients, as indicated by their blood CD4 cell counts, affected detection rates or absolute titres of HIV-1 proviral DNA in semen and blood. Also, in the subset of patients for whom semen cell counts were available, detection of HIV-1 provirus was associated with increased semen white blood cell count, and more specifically with increased numbers of HIV-1 host cells, particularly macrophages, although this association was not statistically significant. These findings are in broad agreement with those described previously [Xu et al., 1997]. By contrast, detection of HIV-1 RNA in semen was independent of disease stage but was associated, albeit not significantly, with increased semen and blood proviral DNA titres and elevated blood viral RNA

TABLE III. Comparison of Medians and Ranges of Blood CD4 Cell Counts and HIV-1 Proviral DNA and Viral RNA Titres

Group	Blood CD4 cell count* (total per μl)	Blood HIV-1 DNA titre† (copies per ml)	Blood HIV-1 DNA titre‡ (copies per 10^5 CD4 blood cells)	Semen HIV-1 DNA¶ titre (copies per ml)	Blood HIV-1 RNA titre** (×10^4 copies per ml)	Semen HIV-1 RNA titre (×10^4 copies per ml)
Blood CD4 cell count <200/μl						
n		15	15	16	18	15
Median		924	1,220	40	2.58	0.87
Range		91–5,678	76–8,867	<6–958	<0.2–97.76	<0.2–47.56
Blood CD4 cell count >200/μl						
n		15	15	15	15	15
Median		469	120	<6	0.68	0.30
Range		9–2,478	2–953	<6–2,171	<0.2–73.31	<0.2–66.78
Semen HIV-1 DNA^{+}						
n	15	15	15		15	14
Median	130	924	1,220		2.02	1.08
Range	40–600	129–5,678	22–8,867		<0.20–61.02	<0.20–66.78
Semen HIV-1 DNA^{-}						
n	16	16	15		17	16
Median	290	329	162		1.70	0.22
Range	50–620	9–256	2–953		<0.20–97.76	<0.2–19.28
Semen HIV-1 RNA^{+}						
n	19	18	18	19	19	
Median	220	542	196	31	6.28	
Range	50–620	42–5,678	11–3,849	<6–2,171	<0.20–97.76	
Semen HIV-1 RNA^{-}						
n	11	11	11	11	11	
Median	190	496	191	<6	0.33	
Range	40–550	9–2,720	2–1,431	<6–71	<0.20–10.09	
Blood HIV-1 RNA^{+}						
n	25	23	22	24		22
Median	160	640	280	27		1.10
Range	10–620	91–5,678	22–8,867	<6–958		<0.20–47.56
Blood HIV-1 RNA^{-}						
n	8	8	8	8		8
Median	295	369	187	<6		<0.20
Range	130–550	9–2,478	2–953	<6–2,171		<0.20–66.78

HIV, human immunodeficiency virus.
Mann Whitney *U*-tests:
*Semen HIV-1 DNA^{+} vs. DNA^{-}, $P < .025$; Blood HIV-1 RNA^{+} vs. RNA^{-}, $P < .1$.
†Semen HIV-1 DNA^{+} vs. DNA^{-}, $P < .025$.
‡Blood CD4 cells >200/μl vs. blood CD4 cells <200/μl, $P < .001$; Semen HIV-1 DNA^{+} vs. DNA^{-}, $P < .005$.
¶Blood CD4 cells >200/μl vs. blood CD4 cells <200/μl, $P < .05$; Semen HIV-1 RNA^{+} vs. RNA^{-}, $P < .05$.
** Semen HIV-1 RNA^{+} vs. RNA^{-}, $P < .01$

titres. Detection of HIV-1 RNA in the semen samples for which the leukocyte cell populations had been determined, was associated with decreased leukocyte and HIV host cell numbers. This result was surprising, particularly given the correlation between semen proviral and viral titres observed for the entire patient set. Therefore, this finding warrants further investigation using a larger number of patients. Of note, the frequency and absolute numbers of leukocyte cell-subsets, even in the small sample size tested, was in very good agreement with those reported for both HIV-1-infected and uninfected individuals [Wolff and Anderson, 1988; Quayle et al., 1997; Xu et al., 1997]. The fact that macrophages and not CD4 lymphocytes constituted the largest population of HIV-permissive cells in semen might in part explain the difference in proviral loads observed here, and the differences in genital tract virus populations compared with blood described elsewhere [Zhu et al., 1996; Gupta et al., 1997; Coombs et al., 1998; Delwart et al., 1998; Eron et al., 1998; Liuzzi et al., 1996; Quayle et al., 1998]. In addition, this might also explain why viruses transmitted sexually are almost entirely of the macrophage tropic, non-syncytium-inducing, phenotype [Wolfs et al., 1992; Zhang et al., 1993; Zhu et al., 1996].

An important aspect of this study was to determine the relationship, if any, between the cell-associated and cell-free virus populations, particularly as this is-

TABLE IV. Comparison of Medians and Ranges of Semen Cell Populations in Patients With Disparate Blood CD4 Cell Counts and Semen HIV-1 Nucleic Acid Status

Group	Leukocytes ($\times 10^5$ ml^{-1})	SFMCs[a] ($\times 10^5$ ml^{-1})	CD4 T cells ($\times 10^5$ ml^{-1})	Macrophages ($\times 10^5$ ml^{-1})	HIV host cells[b] ($\times 10^5$ ml^{-1})
Blood CD4 cell count <200/μl (n = 10)					
Median	3.48	2.49	0.07	1.09	1.70
Range	0.22–23.31	0.18–18.27	ND–1.23	0.09–4.07	0.09–4.93
Blood CD4 cell count >200/μl (n = 9)					
Median	4.40	2.08	0.31	1.24	1.55
Range	0.05–34.72	0.01–21.27	ND–7.09	0.02–4.96	0.02–10.42
Semen HIV-1 DNA$^+$ (n = 8)					
Median	6.10	4.53	0.50	1.62	2.76
Range	0.11–23.31	0.06–18.27	ND–1.23	0.04–4.07	0.04–4.93
Semen HIV-1 DNA$^-$ (n = 11)					
Median	2.62	1.52	ND	1.01	1.01
Range	0.05–34.72	0.01–21.27	ND–7.09	0.02–4.96	0.02–10.42
Semen HIV-1 RNA$^+$ (n = 10)					
Median	1.31	0.76	0.07	0.58	0.65
Range	0.11–34.72	0.06–21.27	ND–7.09	0.04–4.96	0.04–10.42
Semen HIV-1 RNA$^-$ (n = 9)					
Median	4.40	3.46	0.31	1.24	2.04
Range	0.05–23.31	0.01–18.27	ND–1.23	0.02–3.15	0.02–3.79

HIV, human immunodeficiency virus; ND, not detected.
[a]Semen mononuclear cells calculated by subtracting the granulocyte cell count from the total leukocyte cell count for each sample.
[b]Calculated by adding the total number of CD4 lymphocytes and macrophages for each sample.

sue had not been addressed in any previous study. Our studies showed positive correlations existed between: (i) semen proviral DNA and semen viral RNA titres; (ii) semen and blood RNA titres; and (iii) semen proviral titres and blood proviral titres. In addition, blood proviral titres were inversely correlated to blood CD4 cell counts. The correlation between blood and semen viral RNA titres and the lack of correlation with blood CD4 cell counts was in agreement with some [Gupta et al., 1997; Xu et al., 1997] but not all previously published studies [Liuzzi et al., 1996; Coombs et al., 1998]. However, in certain individuals, disparate titres of provirus and virus within the two compartments was observed, indicating that HIV-1 can coexist as distinct subpopulations. Recently, Coombs et al. [1998] showed that semen viral RNA titres were correlated with culturable HIV-1 in the cellular fraction of semen, and our data would indicate that this correlation might be due principally to increasing proviral titres per se. This finding also indicates that observations of increased sexual transmission rates associated with elevated viral RNA [Lee et al., 1996] might be due to a corresponding increase in semen cell-associated virus. Therefore, the exact role of cell-associated and cell-free virus in the transmission process will require further clarification.

ACKNOWLEDGMENTS

The authors thank Dr. C. Bignall, Dr. I. Ahmed, Dr. C. Bowman, Dr. S. Drake, and colleagues for the provision of clinical material, and P. McClure for his technical assistance.

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